Biosynthesis of the Zaragozic Acids. 1. Zaragozic Acid A

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The biosynthetic precursors of zaragozic acid A, a member of a new class of squalene synthase inhibitors, were determined. Washed cells of the producing culture, an unidentified sterile fungus, were supplemented with both ^{14}C- and ^{13}C-labeled substrates, and isolated zaragozic acid A was analyzed by scintillation counting and carbon NMR analysis, respectively. Zaragozic acid A is derived from two polyketide chains. Chain A begins with an aromatic ring derived from benzoic acid, itself derived from metabolism of phenylalanine, and continues in a polyketide fashion by condensation of five acetate units. The terminal four carbon atoms of chain A appear to arise from condensation of succinic acid to the polyketide chain, although citric acid cannot be ruled out as a source of the terminal six carbons. The acetyl group at C-4′ arises from acetate, and the final two carbons of chain A arise by C-methylation from the methyl of L-methionine to give the methylene at C-3′ and the methyl at C-5′. Chain B is a polyketide formed by condensation of four acetate units and two C-methylations from L-methionine to give the two branched methyl groups at C-4″ and C-6″. Proposals for the biosynthesis of related products zaragozic acids B and C are presented.

The zaragozic acids (Figure 1) are a recently discovered family of structurally unprecedented fungal metabolites that exhibit picomolar inhibitory activity in vitro against rat squalene synthase.[1-7] The zaragozic acids also display broad spectrum activity against fungi at concentrations as low as 0.25 μg/mL.[1-4] Three of the principle members of the zaragozic acid family are zaragozic acids A, B, and C (Figure 1). Zaragozic acid A is produced by an unidentified sterile fungus (MF5453), while zaragozic acids B and C are produced by *Sporormiella intermedia* and *Leptodontium elatius*, respectively. The zaragozic acids share the common chemical feature of a novel 4,6,7-trihydroxy-2,8-dioxobicyclo[3.2.1]octane-3,4,5-tricarboxylic acid ring system. Differences among the zaragozic acids arise from variation of the 6-*O*-acyl and 1-alkyl side chains. The absolute stereochemistry of zaragozic acid A was recently determined using a combination of degradation and spectroscopic and crystallographic procedures.[6] A group of compounds called the squalestatins, which appear to be identical to zaragozic acid A, 4′-desacetylzaragozic acid A and 6-*O*-desacylzaragozic A acid, were recently reported to be produced by a strain of the fungus *Phoma*.[8,9]

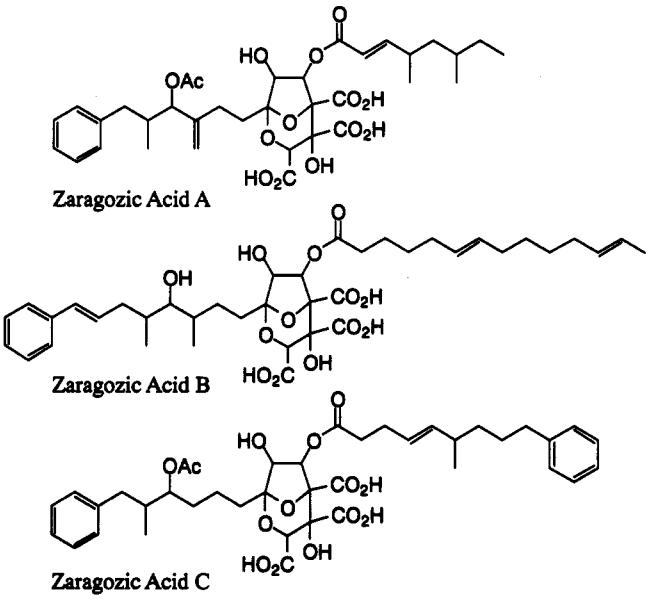

Figure 1. The structures of the zaragozic acids.

The zaragozic acids represent a new class of biologically active compounds containing an aromatic group, two polyketide chains, and a unique, highly oxidized, bicyclic ring system substituted with three carboxylic acid groups. The novel structural class comprising the zaragozic acids makes their biosynthesis of particular interest. Knowledge of the biosynthetic precursors could be useful for generating new structural analogues through directed biosynthetic techniques. This paper presents results of ^{14}C- and ^{13}C-labeling experiments performed with washed cells of the zaragozic acid A producing fungus to elucidate the biosynthetic precursors of zaragozic acid A.[10] Because of the common features shared by all three zaragozic acids, the biosynthesis determined for zaragozic acid A was used

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Table I. Incorporation of Carbon-14 Labeled Precursors into Zaragozic Acid A

substrate[a]	μCi precursor added/L	sp act. of precursor (μC/μmol)	sp act. zaragozic acid (μC/mmol)	% incorporation
[2-^{14}C]acetic acid	321.5	3.21	32.7	1.10
[2-^{14}C]propionic acid	176.0	1.76	11.0	0.70
L-[*methyl*-^{14}C]methionine*	265.6	46.0	148.0	5.90
L-[U-^{14}C]aspartic acid	249.0	2.49	14.6	1.31
[1,5-$^{14}C_2$]citric acid	257.5	2.58	9.5	1.54
[2,3-$^{14}C_2$]succinic acid	264.5	2.65	27.1	2.43
L-[U-^{14}C]tyrosine	212.0	2.12	6.3	0.14
L-[U-^{14}C]phenylalanine	197.6	1.98	87.6	12.90
L-[1-^{14}C]phenylalanine	201.2	2.01	11.2	1.60
L-[5-^{3}H]trytophan	696.0	6.96	8.5	0.05
[7-^{14}C]benzoic acid	180.8	1.81	69.4	4.12
[7-^{14}C]benzoic acid*	169.2	15.0	149.3	10.38
[*ring*-^{14}C]cinnamic acid	63.6	0.63	134.4	13.74
[U-^{14}C]shikimic acid	220.0	2.20	61.6	0.68

[a] All radioactive precursors, except where denoted by an asterisk (*), were diluted with cold compound to achieve a final concentration of 0.1 mM in the flasks/tubes. Substrates marked with an asterisk (*) were not diluted with cold compound, but used directly as supplied by the manufacturer.

as the basis to propose the biosynthetic precursors of zaragozic acids B and C.

Results

Proposed Biosynthesis. Our hypothetical model for the biosynthesis of zaragozic acid A called for synthesis from two polyketide chains: chain A to form the alkyl side chain and bicyclic ring core, while chain B would provide the acyl side chain. Chain A would commence with a starter unit consisting of an activated form of shikimic acid or an aromatic amino acid metabolite. Extension of chain A could occur either by condensation of acetate units via malonyl CoA and C-methylation from *S*-adenosylmethionine (SAM), or alternatively by condensation of acetate and propionate units arising from malonyl- and methylmalonyl-CoA's, respectively. The terminal units of chain A giving rise to the bicyclic ring atoms are likely to be derived from triose pathway metabolites, such as glycerate or glycolate, coupled with an activated form of a citric acid cycle intermediate, e.g. succinate or citrate. We expected that the biosynthesis of polyketide chain B would utilize acetate as a starter unit and proceed either by condensation of two propionates/methyl malonates and one acetate, or alternatively by three acetates/malonates and two C-methylations utilizing SAM as methyl donor.

Incorporation of Acetate, Propionate and Methionine. The ability of MF5453 to incorporate acetate, propionate, and methionine into zaragozic acid A was first examined using ^{14}C-labeled substrates added to washed cell incubations (Table I). Acetate was incorporated into zaragozic acid A at 1.1% in an incubation medium containing 60 g/L sucrose. In the absence of an appropriate carbon source, production of zaragozic acid A was negligible. The weak propionate incorporation of 0.7%, coupled with the much higher incorporation of methionine at 5.9%, suggested that if propionate was being incorporated at all, it would only be at a limited number of possible sites. Methionine was the likely precursor of several of the branched methyl/methylene carbon atoms. Feeding of [1-^{13}C]-, and [1,2-$^{13}C_2$]acetic acid identified the direction and position of 10 acetate units in zaragozic acid A (Figure 2). Four acetate units provide the entire backbone of the acyl side chain (chain B). Five acetate units form a continuous acetate backbone extending from carbon 5′ in the alkyl side chain (chain A) through the carboxylic acid carbon (C-10) attached to carbon 5 of the bicyclic ring. The final acetate unit gives rise to the C-4′ acetyl group (Figure 2). When washed cells were supplemented with [1-^{13}C]acetic acid, the relative enrichment values at the enriched carbon sites were quite uniform in both chains A and B, except for the two ester carbons, C-15′ and C-1″ (Table II). The smaller relative enrichment values associated with these two carbons were attributed to the inherently poor signal intensity of quaternary carbon atoms.

In the spectrum obtained of zaragozic acid A produced in the presence of [1,2-$^{13}C_2$]acetic acid, all 20 acetate-derived carbon atoms showed equivalent enrichment. More importantly, all 20 signals exhibited the characteristic doublet that results when doubly labeled acetate is incorporated into the molecule as a single unit. Of particular note was the coupling observed between carbons C-5 and C-10, the terminal acetate unit of chain A. While significant enrichment at C-10 was not detected in the spectrum of zaragozic acid A produced in the presence of [1-^{13}C]acetic acid, relative enrichments of 4–6 were associated with the doublets at C-5 and C-10 following supplementation with [1,2-$^{13}C_2$]acetate. Bicyclic ring carbon atoms 3, 4, 8, and 9 showed much weaker (1.5–2.0) relative values in the same spectrum and exhibited no significant coupling. This finding indicates that the labeled acetate was further metabolized (e.g. catabolism of acetate-derived carbohydrate, lipid, and amino acids back into and through the citric acid cycle) with concomitant dilution and scrambling of the labeled precursor. Such a result is consistent with our biosynthetic model proposing that this portion of the molecule is derived from a citric acid intermediate.

Carbon NMRs of zaragozic acid A produced in the presence of [1-^{13}C]propionic acid showed no specific sites of relative enrichment greater than 2. However, supplementation with L-[*methyl*-^{13}C]methionine resulted in high levels of enrichment (20–25-fold) at all four branched chain carbon sites, C-9″, -10″, -13′, and -14′ (Table II). This indicates that the three branched chain methyl/methylene groups on the acyl and alkyl side chains of zaragozic acid A are all derived from the methyl group of L-methionine. These results also fully corroborate the previously discussed ^{14}C incorporation data which showed excellent incorporation of L-methionine and weak incorporation of propionate into zaragozic acid A.

Incorporation of Citric Acid Cycle Intermediates. Utilization of citric acid cycle intermediates by MF5453 for the biosynthesis of zaragozic acid A was first examined using ^{14}C-labeled aspartic (expected to be metabolized to

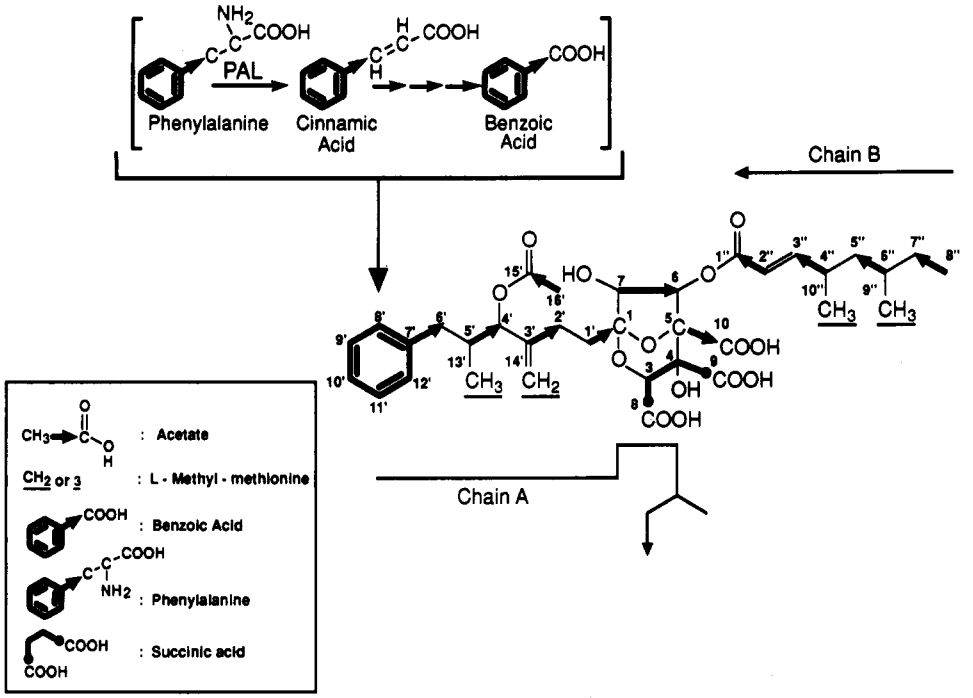

Figure 2. Labeling patterns of zaragozic acid A from [1-^{13}C]- and [1,2-$^{13}C_2$]acetate, L-[*methyl*-^{13}C]methionine, DL-[3-^{13}C]phenylalanine, [*carboxy*-^{13}C]- and [*ring*-$^{13}C_6$]benzoic acid, and [1,4-$^{13}C_2$]-, [2,3-$^{13}C_2$]-, and [1,2,3,4-$^{13}C_4$]succinic acid.

Table II. Incorporation of ^{13}C-Labeled Precursors into Zaragozic Acid A

substrate	carbon atom[a]	relative ^{13}C enrichment[b]
[1-^{13}C]acetic acid	C: 15′, 1″	2.0–3.0
	C: 1, 6, 2′, 4′, 3″, 5″, 7″	4–8
[1,2-$^{13}C_2$]acetic acid	C: 1″-2″, 3″-4″, 5″-6″, 7″-8″	4–6
	C: 5′-4′, 3′-2′, 1′-1, 15′-16′	4–6
	C: 6-7, 5-10	4–6
	C: 3, 4, 8, 9 (not coupled)	1.5–2.0
L-[^{13}C-methyl]methionine	C: 13′, 14′, 9″, 10″	20–25
DL-[3-^{13}C]phenylalanine	C-6′	50–100
[*carboxyl*-^{13}C]benzoic acid	C-6′	40–50
[3-^{13}C]oxaloacetate[c]	C: 3, 4, 5	1.8–2.0
	C: 3′, 4′, 7′, 1″, 2″	1.5–1.8
	C: 1, 7, 8, 9, 10	1.5–1.8
[4-^{13}C]aspartic acid[c]	C-8	2.0
	C: 1, 9, 3′, 4′, 7′, 15′, 1″	1.5–2.0
[1,4-$^{13}C_2$]succinic acid[c]	C-8	6
	C-9	6
[2,3-$^{13}C_2$]succinic acid	C: 3–4	4–9[d]
[1,2,3,4-$^{13}C_4$]succinic acid	C: 8–3–4–9	6–18[d]
[*ring*-$^{13}C_6$]benzoic acid	C: 7′, 8′, 9′, 10′, 11′, 12′	40–60
[2,3,4-$^{13}C_3$]citric acid	C: 3–8, 4–9, (3–4)	1.20–1.25
	C: 1″–2″, 3″–4″, 5″–6″, 7″–8″	1.05–1.10
	C: 5′–4′, 3′–2′, 1′–1, 5–10, 6–7	1.05–1.10

[a] Carbon numbers separated by a dash exhibited carbon–carbon coupling. Carbon coupling constants (J_{cc}) of zaragozic acid A obtained from succinate feedings were $J_{3,4}$ = 37.0 Hz from [2,3-$^{13}C_2$]succinic acid, and $J_{3,4}$ = 36.6 Hz, $J_{3,8}$ = 64.1 Hz, and $J_{4,9}$ = 62.4 Hz from [1,2,3,4-$^{13}C_4$]succinic acid; citrate feedings gave $J_{5,10}$ = 75 Hz, doublets of 64.5 and 61.8 Hz, C-8 and C-9, respectively, and complex multiplets at C-3 and C-4. [b] See the Experimental Section. [c] These substrates were supplemented to cells aliquoted from the same batch, added and grown under identical conditions. [d] These relative enrichment values represent incorporation of [2,3-$^{13}C_2$]- and [1,2,3,4-$^{13}C_4$]succinic acids into zaragozic acid A as intact molecules and disregards enrichment due to scrambling. Only those peak areas corresponding to the appropriate doublets or doublet of doublets were included in enrichment calculations.

the citric acid cycle intermediate oxaloacetic acid),[11] citric, and succinic acids (Table I). [2,3-^{14}C]Succinic acid was found to be incorporated at nearly twice the level as either [1,5-^{14}C]citric or L-[U-^{14}C]aspartic acids. The 2-fold better incorporation of succinic acid versus citric acid is consistent with citric acid metabolism to succinic acid, wherein one of the two ^{14}C-labeled atoms of [1,5-^{14}C]citric acid would be lost as CO_2. This data suggests that succinic acid is a more proximal precursor of zaragozic acid A than citric or oxaloacetic acids, if the uptake of the acids is roughly equivalent.

The biosynthetic precursor of the final four carbons of chain A (C-8, -3, -4, and -9) was further explored using ^{13}C-labeled intermediates of the citric acid cycle. Supplementations were performed with [3-^{13}C]oxaloacetate, [4-^{13}C]aspartic acid, [2,3,4-$^{13}C_3$]citric acid, and succinic acid labeled in various positions. The highest levels of enrichment were obtained with the succinic acid substrates. If succinate either serves as the direct precursor or is

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incorporated through metabolism to citric acid, then [1,4-$^{13}C_2$]succinate should label C-8 and C-9 of zaragozic acid A, [2,3-$^{13}C_2$]succinate should label C-3 and C-4, and [1,2,3,4-$^{13}C_4$]succinate should label C-8, -3, -4, and -9. The results of these succinate supplementations are presented in Table II. [1,4-$^{13}C_2$]Succinate was incorporated into the bicyclic ring with a relative enrichment of 6 at the predicted sites C-8 and C-9. Likewise, [2,3-$^{13}C_2$]- and [1,2,3,4-$^{13}C_4$]-succinic acids were both well incorporated and produced substantial enrichments at C-3/C-4 and C-8/C-3/C-4/C-9, respectively. Furthermore, the splitting pattern in the spectra obtained after these latter two feeding experiments provided direct evidence that succinic acid was incorporated as an intact unit into carbons 8, 3, 4, and 9. A strong doublet ($J_{3,4}$ = 37.0 Hz) at the C-3 and C-4 resonances appeared in the spectrum resulting from [2,3-$^{13}C_2$]-succinate supplementation, while [1,2,3,4-$^{13}C_4$]succinate supplementation produced pronounced doublets at C-8 ($J_{3,8}$ = 64.1 Hz) and C-9 ($J_{4,9}$ = 62.4 Hz) resonances, and dominant doublet of doublets at both C-3 and C-4 ($J_{3,4}$ = 36.6 Hz, $J_{3,8}$ and $J_{4,9}$ as above) resonances.

Attempts to label zaragozic acid A with the other ^{13}C-labeled intermediates of the citric acid cycle were less successful. Significantly lower levels of enrichment (2.0 or less) were obtained with [3-^{13}C]oxaloacetate, [4-^{13}C]-aspartic acid, and [2,3,4-$^{13}C_3$]citric acid. The labeling patterns derived from supplementation of these compounds indicated metabolism in both directions of the citric acid cycle, as well as significant metabolism to acetate. The most striking feature of the NMR pattern arising from the low level incorporation (relative enrichment averaged 1.15) of [2,3,4-$^{13}C_3$]citric acid was the appearance of doublets at all acetate derived carbon atoms, indicating metabolism of the substrate to [1,2-$^{13}C_2$]acetate.

Incorporation of Aromatic Precursors. The origin of the aromatic ring of zaragozic acid A was explored using ^{14}C-labeled precursors. [U-^{14}C]Shikimic acid was incorporated into zaragozic acid A at 0.68% (Table I), a level that suggested the existence of a more direct precursor. Subsequent results obtained with L-[U-^{14}C]phenylalaine and L-[U-^{14}C]tyrosine clearly indicated the preference for L-phenylalanine incorporation into zaragozic acid A with 12.9% incorporation. Since the ^{13}C-acetate labeling pattern discussed above identified 5 of the 12 carbons of the alkyl side chain as arising from 3 acetate units, the 9 carbons of phenylalanine would have to be metabolized to a compound that could provide the remaining 7 carbons of the alkyl side. This proposal was investigated using phenylalanine labeled with ^{14}C and ^{13}C in various positions. Incorporation of DL-[3-^{13}C]phenylalanine resulted in a relative enrichment value greater than 50 specifically at C-6′. The level of incorporation of ^{14}C-labeled phenylalanine was found to be dependent upon the position of label in the substrate. While L-[U-^{14}C]phenylalanine was incorporated into zaragozic acid A at 12.9%, L-[1-^{14}C]-phenylalanine was incorporated at only 1.6%. These results strongly suggested metabolism of phenylalanine to a benzoic acid type precursor, quite possibly involving the common fungal enzyme phenylalanine ammonia lyase (PAL). This enzyme converts phenylalanine to cinnamic acid followed by cleavage of an acetate unit to generate benzoic acid.

Additional support for the involvement of this pathway was obtained by feeding [7-^{14}C]benzoic acid and [*ring*-^{14}C]cinnamic acid to washed cells of MF5453. Benzoic acid was incorporated into zaragozic acid A at a level of 10.4%, while cinnamic acid was incorporated at 13.7%. The site of incorporation of benzoic acid was established using both [*carboxyl*-^{13}C]- and [*ring*-^{13}C]benzoic acids. A relative enrichment of 40–50 at C-6′ was obtained when [*carboxyl*-^{13}C]benzoic acid was incorporated. Feeding [*ring*-^{13}C]benzoic acid led to a relative enrichment of 40–60 at the aromatic carbon resonances. The incorporation of ^{14}C-labeled benzoic acid and phenylalanine, taken together within the incorporation of ^{14}C-labeled cinnamic acid, provide strong evidence for a pathway leading from phenylalanine through cinnamic acid to benzoic acid and incorporation into zaragozic acid A (Figure 2). An interesting feature of the carbon NMR spectrum of zaragozic acid A produced in the presence of [*ring*-^{13}C]-benzoic acid was the satellite doublets appearing at each of the carbon atoms that were derived from acetate. With relative enrichments at the enriched sites of 2.0–3.0, the spectrum rivaled that observed in the spectrum obtained following incorporation of [1,2-$^{13}C_2$]acetate. This result strongly suggests that MF5453 can metabolize the aromatic carbons of benzoic acid to acetate, possibly through catechol or protocatechuic acid.

Discussion

All ^{14}C and ^{13}C data obtained in the described experiments confirmed the two-chain proposal for zaragozic acid biosynthesis (Figure 2). Chain A begins with the aromatic ring on the alkyl side chain. These six carbon atoms, along with the adjacent benzylic carbon (C-6′), arise from catabolism of phenylalanine through cinnamic acid to benzoic acid. The most probable route for this conversion involves the action of phenylalanine ammonia lyase to generate cinnamic acid, followed by β-oxidation and truncation to give acetate and benzoic acid.[12] The presence of PAL in the producing culture has been demonstrated,[13] and PAL inhibitors phenylpropiolic acid and D-phenylalanine are both strong inhibitors of zaragozic acid A production (data not shown). The actual starter molecule of chain A is likely an activated form of benzoic acid, presumably benzoyl CoA, analogous to the activated form of 1-cyclohexenecarboxylic acid utilized in ansatrienin biosynthesis.[14] Chain A is extended by addition of five acetate units, presumably in the activated form of malonyl CoA's. Chain A terminates with the final addition of a succinate molecule, again likely as succinyl CoA. Succinate has been established as a direct precursor in the biosynthesis of a number of secondary metabolites.[15-17] Chain B is formed and extended in a straight-chain polyketide fashion using four acetate/malonate units. Branched chain methyl groups on both chains A and B do not arise by condensation of a one carbon higher homologue (i.e. propionate in place of acetate) as is typical in polyketides made by streptomycetes, but rather through a C-methylation reaction utilizing methionine as the methyl donor.

We propose succinate to be the precursor of carbons 3, 4, 8, and 9 in the biosynthesis of zaragozic acid A, and that

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carbons 5 and 10 arise from one acetate unit. Neither our data nor that recently published elsewhere[10] can exclude the possibility that these six carbons all arise directly from citric acid. Our data also does not exclude oxidation of succinate to an analogue of a citric acid intermediate (e.g. epoxyfumarate), before incorporation into zaragozic acid A. Several pieces of evidence do, however, favor succinate over oxaloacetic acid or citric acid as a more proximal precursor for carbons 8, 3, 4, and 9. First, incorporation of [1,2-^{13}C]acetate into C-5 and C-10 were comparable in enrichment to those in the rest of chain A. One might expect to see lower enrichment values for C-5 and C-10 if acetate were being diluted by the endogenous citric acid pool prior to incorporation. Secondly, [1,4-$^{13}C_2$]succinate was incorporated into the expected positions, C-8 and C-9, with little enrichment at other sites. Competing with this use of succinate was conversion to acetate, presumably through established pathways from malate and oxaloacetate to pyruvate. Such a conversion was apparent in spectra obtained following supplementation of washed cells with [2,3-$^{13}C_2$]- and [1,2,3,4-$^{13}C_4$]succinate in which relative enrichments of roughly 2.0 were observed in the form of doublets at all acetate-derived carbon atoms. Despite the competing metabolism to acetate, relative enrichments of 4–9 and 6–18 were observed at the expected sites for [2,3-$^{13}C_2$]succinate and [1,2,3,4-$^{13}C_4$]succinate, respectively (Table II). This was not true when cells were supplemented with [3-^{13}C]oxaloacetic acid. While one could attribute the weak relative enrichments of 2.0 to poor uptake, the fact that carbons derived from acetate in both alkyl and acyl side chains were enriched to roughly the same extent as the carbons in the bicyclic ring suggests some uptake and metabolism of the substrate. Scrambling of the label through symmetrical fumarate to succinate and back to oxaloacetic acid, in addition to the known pathways to acetate, would account for the majority of the weakly enriched sites. A similar finding was observed when cells were supplemented with [4-^{13}C]aspartic acid, a precursor of oxaloacetic acid.

Finally, supplementation of washed cells with [2,3,4-$^{13}C_3$]citric acid led to weak (relative enrichments of 1.05–1.10) but discernible doublets at almost all acetate-derived carbon atoms. Larger relative enrichment values were associated with the pattern of complex multiplets which appeared at carbons 3 and 4, while doublets with relative enrichments of 1.20–1.25 occurred at C-8 and C-9. In comparison, the doublets at C-5 and C-10 were similar in intensity to the acetate-derived carbons with relative enrichments of 1.07–1.09. The enrichments at carbons 3, 4, 8, and 9 are consistent with metabolism of [2,3,4-$^{13}C_3$]-citrate to [1,2,3-$^{13}C_3$]succinate, while the weaker enrichments at the acetate derived carbons represent further metabolism through pyruvate to [1,2-$^{13}C_2$]acetate.

All of the principal zaragozic acids contain the same bicyclic ring system with an acyl side chain at C-6 and an aromatic ring containing alkyl side chain at the C-1. Variation among family members arises from changes in the acyl and alkyl side chains. The structural similarity of this family of compounds allows one to speculate on the biosynthesis of zaragozic acids B and C (Figure 1). Zaragozic acid B lacks the acetate-derived acetyl group at C-4′ and contains two additional carbons in the alkyl side chain that should arise from addition of one acetate unit. Whereas the acyl side chain in zaragozic acid A arises from condensation of four acetate/malonate units, that of zaragozic acid B would probably arise from seven acetate/malonate units with no methyl branches from L-methionine. The alkyl side chain of zaragozic acid C is equivalent to that of zaragozic acid A, lacking only the C-methylation from L-methionine at C-3′. The acyl side chain of zaragozic acid C exhibits the greatest diversity among the zaragozic acids currently identified. It contains a single methyl branched polyketide chain that begins with an aromatic ring, as in the case of the alkyl side chains. One might expect the organism to utilize similar biosynthetic enzymes for both side chains, suggesting the acyl side chain would arise by condensation of one benzoate and four acetate/malonates with a methyl branch arising from L-methionine.

Experimental Section

Strains and Media. Zaragozic acid A producing culture, MF5453 (ATCC 20986), was stored as frozen vegetative mycelia in seed medium containing 10% glycerol. Seed medium consisted of tomato paste, 40 g/L; corn steep liquor, 5 g/L; D-glucose, 10 g/L; oat flour, 10 g/L; and 10 mL/L of a trace mineral mixture composed of $FeSO_4 \cdot 7H_2O$, 1.0 g; $MnSO_4 \cdot 4H_2O$, 1.0 g; $CuCl_2 \cdot 2H_2O$, 0.025 g; $CaCl_2 \cdot 2H_2O$, 0.1 g; H_3BO_3, 0.056 g; $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 0.019 g; and $ZnSO_4 \cdot 7H_2O$, 0.2 g, dissolved in 1 L of 0.6 N HCl, and adjusted to pH 6.8 before autoclaving. Growth was initiated by inoculating 50 mL of seed medium in a 250-mL nonbaffled Erlenmeyer flask with 1.5 mL of frozen vegetative cells and incubating the culture at 220 rpm and 25 °C for 48–72 h.

Washed Cell Incubations. Vegetative cultures were harvested at 48–72 h, centrifuged, resuspended to the original volume in distilled water, and homogenized with a hand-held biohomogenizer (Biospec Products, Bartlesville, OK) until large clumps disappeared (ca. 20 s, low speed). The mycelia were washed once more with distilled water and then resuspended to the original volume. A volume of resuspended cells (1.25 mL in 25- × 150-mm tubes, 15 mL in 250-mL Erlenmeyer flasks, or 150 mL in 2000-mL flasks) was added to an equal volume of incubation buffer containing 30–60 g/L sucrose and 20 mM of either MES or PIPES buffer, pH 6.1. Sucrose at 30 g/L was used when incorporating ^{13}C-labeled citric acid cycle intermediates. The resuspended mycelia were incubated at 25 °C and 220 rpm for 72–96 h. Precursors were adjusted to pH 5.0–6.0 prior to addition to washed and resuspended cells. Radioactive precursors were added to the washed cells when production of zaragozic acid A was between 10 and 50 μg/mL (42–48 h). Cells were harvested after 72–96 hours. ^{13}C-Labeled substrates were added to the washed cells at concentrations of 0.3–3.0 g/L and added in four equal additions, at 30, 48, 54, and 72 h.

Product Isolation and Purification. Zaragozic acid A was isolated in crude form by the addition of two volumes of MeOH per volume of washed cell incubation previously adjusted to pH 2.8 with formic acid. The extracted mixture was vigorously shaken for 30 min and centrifuged, and the supernatant was removed. The MeOH was evaporated under vacuum and the resulting aqueous mixture extracted with an equal volume of ethyl acetate. The ethyl acetate was removed under vacuum and the resulting residue dissolved in acetonitrile/0.1% H_3PO_4 (60:40). Zaragozic acid A isolated from supplementations with ^{13}C-labeled compounds was further purified by preparative HPLC on a 21.4-mm × 25-cm Rainin Dynamax-60A C18 column equilibrated at 40 °C and eluted in acetonitrile/0.1% H_3PO_4 (55:45) at a flow rate of 10 mL/min and detected at 210 nm. The fractions containing zaragozic acid A were pooled, acetonitrile was stripped under vacuum, and zaragozic acid A was extracted into ethyl acetate. The ethyl acetate fraction was dried over anhydrous sodium sulfate, filtered, and dried, affording pure zaragozic acid A.

Carbon-14 Incorporation. Incorporation of ^{14}C-labeled compounds was determined by analysis of the crude zaragozic acid A on an analytical HPLC column. An aliquot of a 10-fold concentrate of crude zaragozic acid A dissolved in HPLC mobile phase was injected onto a Whatman Partisil C8 column equilibrated at 50 °C in a mobile phase of acetonitrile/0.1% H_3PO_4 (58:42), with a flow rate of 1.0 mL/min, and fractions were

collected into scintillation tubes. The radioactivity in each fraction was determined in a Beckman Model 5801 liquid scintillation counter. The titer of zaragozic acid A in each fraction was determined by comparison of peak area of the sample measured at 210 nm to pure zaragozic acid A as external standard.

Radioisotopes. [2-^{14}C]Acetate (60 mCi/mmol) and [2-^{14}C]-propionate (55.7 mCi/mmol) were purchased from ICN Biomedicals; [7-^{14}C]benzoic acid (15 mCi/mmol), L-[*methyl*-^{14}C]-methionine (46 mCi/mmol), [U-^{14}C]shikimic acid (21.9 mCi/mmol), and L-[5-^{3}H]tryptophan (24.5 mCi/mmol) were purchased from DuPont/NEN; and L-[U-^{14}C]aspartic acid (206 mCi/mmol), [*ring*-U-^{14}C]cinnamic acid (3.5 mCi/mmol), [1,5-^{14}C]citric acid (110 mCi/mmol), L-[U-^{14}C]phenylalanine (475 mCi/mmol), L-[1-^{14}C]phenylalanine (55 mCi/mmol), [2,3-^{14}C]succinic acid (56 mCi/mmol), and L-[U-^{14}C]tyrosine (457 mCi/mmol) were purchased from Amersham Corp.

Stable Isotopes. [1-^{13}C]Acetic acid, Na salt, L-[4-^{13}C]aspartic acid, [*carboxyl*-^{13}C]benzoic acid, [2,3,4-$^{13}C_3$]citric acid, L-[*methyl*-^{13}C, 96% ^{13}C]methionine, [3-^{13}C]oxaloacetate, [1-^{13}C]propionic acid, Na salt, [1,4-$^{13}C_2$]succinic acid, [2,3-$^{13}C_2$]succinic acid, and [1,2,3,4-$^{13}C_4$]succinic acid were purchased from Cambridge Isotope Laboratories; DL-[3-^{13}C]phenylalanine was purchased from Isotec, Inc.; [1,2-$^{13}C_2$]acetic acid, Na salt, was purchased from Sigma Chemical Co. All ^{13}C isotopes were 99% ^{13}C except as noted.

NMR Spectroscopy. The ^{13}C NMR spectra were acquired in CD_3OD on a Varian Unity 500 spectrometer operating at 125 MHz. Chemical shifts are given in ppm relative to CD_3OD at 49.0 ppm as internal standard. Sites of enrichment were based upon the following established shift assignments,[5] in ascending order: core C-1 to C-10 are 106.9, 76.8, 75.7, 91.3, 81.3, 82.8, 170.3, 172.7, 168.7; alkyl side chain C-1′ to C-7′ are 35.2, 26.7, 147.9, 80.4, 37.9, 41.0, 141.7; C-8′/12′ is 130.3; C-9′/11′ is 129.4; C-10′ is 127.0; C-13′ to C-16′ are 14.3, 111.7, 172.2, 20.9; acyl side chain C-1″ to C-10″ are 166.7, 120.0, 157.6, 35.6, 44.5, 33.2, 30.9, 11.5, 19.3, 20.6 ppm. Relative ^{13}C enrichments were expressed as the ratio of the intensity of each peak in the labeled product divided by the intensity of the same peak in the natural abundance spectrum, normalized to give a ratio of 1.0 relative to selected unenriched peaks {C-7′ for the [*methyl*-^{13}C]methionine derived product, all others normalized to an average intensity for carbons C-13′, C-9″, and C-10″}. Every effort was made to perform NMR analyses under uniform conditions. However, in view of the nonquantitative nature of ^{13}C NMR measurements,[18] relative ^{13}C enrichments less than 2.0 were not considered significant and are not reported in Table II. Only in specific cases where the uniqueness of the enrichment, e.g. coupled signals, or comparison to carbons of similar biosynthetic origin, were relative enrichment values less than 2.0 included.

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(18) Shoolery, J. N.; Jamkowski, W. C. *Quantitative Aspects of ^{13}C NMR Spectroscopy*; Varian Associates: Palo Alto, CA, 1973; Publication No. NMR-73-4; pp 1–18.